Supplementary information

Supplementary materials and methods

Cell line

HyH12.6 (A^k restricted), specific for hen egg lysozyme (HEL) was gift from Prof. Peter Walden (Humbolt University, Charite', Berlin, Germany).The T cell hybridoma LMR7.5 (A^d restricted), specific for 156-173 sequence of Leishmania homolog of receptors for activated C-kinase ($LACK_{156-173}$ peptide) was gift from Prof. Eveylene Mougneau. Chinese hamster Ovary (CHO) cell used for transfection with A^k and mutant A^k. The cell lines were maintained in RPMI-1640 medium supplemented with 10% FCS and 2-ME (5×10^{-5} M) at 37°C with 5% CO_2 in a humidified atmosphere.

Animals

BALB/C mice were obtained from the animal facility of the institute and CBA/J mice were procured from National Institute of Immunology (New Delhi, India). Animals were used for experimental purposes with prior approval of the institutional animal ethics committee. Mice were housed under conventional conditions, with food and water adllibitum.

Peptide Synthesis and characterization

156-173 amino acid residues (ICFSPSLEHPIVVSGSWDR) of LACK protein (defined as $LACK_{156-173}$) (A^d restricted), 46-61 amino acid residues (NTDGSTDYGILQINSR) of HEL protein (defined as HEL_{46-61}) (A^k restricted), heterodimeric transmembrane domain of MHC II (TM) and mutant TM peptides were synthesized in solid phase via Fmoc chemistry on Rink amide PEGA resin in a peptide synthesizer manufactured by Protein Technologies, Inc. (AZ, USA).

The α chain of transmembrane helix (α TM II) and β chain of transmembrane helix (β TM) of MHC II were synthesized by standard solid phase Fmoc chemistry. The TM (contains both α and β chains) were synthesized using orthogonally protected [Fmoc-Lys(Dde)]. The α chain and β chain of transmembrane helix were coupled using Lys as linker amino acid. The α chain of TM helix was synthesized at the α amine of Fmoc-Lys(Dde) and to block the terminal amine of the α chain (residue T219) acetic anhydride was used. Then the Dde group

cleaved with 2% hydrazine and the β chain of TM helix was synthesized at the ε amine. To increase the solubility of the branch heterodimeric peptide six Lys added at the N-terminal end of the β chain. The mutant TM was synthesized by similar method. The mutant amino acids are in green color (Figure 4B).

The peptides were cleaved from the resin using 82.5% TFA, 5% thioanisole, 5% phenol, 5% water, 2.5% ethane dithiol and purified by reverse phase HPLC. Peptides were characterized by mass spectroscopy in ABI-4800 MALDI-TOF-TOF mass spectrometer.

Quantification of membrane cholesterol with an Amplex Red assay kit.

The cholesterol content was determined by using an Amplex Red reagent kit **(1)** and the results were expressed in μM cholesterol/10 μg protein.

Measurement of fluorescence anisotropy (FA)

The membrane fluidity of cells were measured following the method described by Shinitzky *et al.* **(2)**. Briefly, the fluorescent probe DPH was dissolved in tetrahydrofuran at 2 mM concentration. To 10 ml of rapidly stirring PBS (pH 7.2), 2 mM DPH solution was added. For labeling, 10^6 cells were mixed with an equal volume of DPH in PBS (C_f 1 μM) and incubated for 2 h at 37°C. Thereafter the cells were washed thrice and resuspended in PBS. The DPH probe bound to the cellular membrane was excited at 365 nm and the intensity of emission was recorded at 430 nm in a spectrofluorometer. The FA value was calculated using the equation: $FA = [(I_{II} - (G \times I_I)]/[I_{II} + (2 \times G \times I_I)]$, where I_{II} and I_I are the fluorescent intensities oriented parallel and perpendicular to the direction of polarization of the excited light **(3)**.

CD Spectroscopy

Far-UV CD spectra were collected on JASCO 500 CD spectrometer with 1mm quartz cuvette at 25°C. Averages of 10 independent scans were taken and the control spectrum containing only the buffer was subtracted from the spectra of each peptide to generate the final data. CD spectra were collected in PBS containing 30% TFE, with final peptide concentration 3 μM. The concentration of peptide was determined by BCA protein assay. The helix content of each peptide was determined from the mean residue elipticity (MRE) at 222 nm, $[\theta]_{222}$ ($mdegcm^2dmol^{-1}$) corrected for the number of amino acids. Percent helicity was

calculated from the ratio $[\theta]_{222}/[\theta]_{max}$, where $[\theta]_{max} = (-44\ 000 + 250T)(1 - k/n) = -23\ 400$ for $k = 4.0$ and $n = 54$ (number of amino acid residues in the peptide) **(4)**.

Stimulation of T cell hybridoma using transfected cells

CHO cells were transfected with either A^k (wild type α chain and wild type β chain) or mutant A^k (wild type α chain and mutant β chain) were used as APCs to stimulate anti-HEL T cell hybridoma (HyH12.6). Briefly, $2.5X10^5$ T cell hybridomas were cocultered with $5X10^4$ appropriate APCs in the presence of HEL_{46-61} peptide. Both T cell and APCs were kept for 24 h in complete medium at 37°C in humified 5% CO2. The resulting culture supernatant was assayed for IL-2 by ELISA.

Statistical variation and presentation

Each experiment was performed three to five times and results are either expressed as means ± standard deviation (SD), or Student's *t* test for significance was performed using Graphpad prism software, and a *P* value of <0.05 was considered to be significant.

References

1. Banerjee, S., Ghosh, J., Sen, S., Guha, R., Dhar, R., Ghosh, M., Datta, S., Raychaudhury, B., Naskar, K., Haldar, A. K., Lal, C. S., Pandey, K., Das, V. N., Das, P., and Roy, S. (2009) *Infect Immun* **77**, 2330-2342
2. Shinitzky, M., and Inbar, M. (1974) *J Mol Biol* **85**, 603-615
3. Shinitzky, M., and Barenholz, Y. (1978) *Biochim Biophys Acta* **515**, 367-394
4. Shepherd, N. E., Hoang, H. N., Abbenante, G., and Fairlie, D. P. (2005) *J Am Chem Soc* **127**, 2974-2983

Supplementary Figure Legend

Figure S1. Alignment showing the conservation of residues comprising the probable cholesterol binding and GxxxG motifs.

The sequence alignment of MHC II chains, α and β (upper and lower panels, respectively), across ten organisms are shown. The sequences corresponding to each organism, their common name and gi-accession numbers for chains, α and β, are: *Mus musculus* (Mouse, gi122197, gi122246), *Canis familiaris* (Dog, gi1208573, gi1209893), *Rattus norvegicus* (Rat, gi54780906, gi54780890), *Sus scrofa* (Pig, gi113197131, gi113197147), *Bos taurus* (Cow, gi1027491, gi1008039), *Ovis aries* (Sheep, gi157862703, gi165931962), *Mastomys natalensis* (African soft-furred Rat, gi254554240, gi254554234), *Homo sapiens* (Human, gi77539960, gi84796221), *Macaca fascicularis* (Cynomolgus Monkey, gi125489002, gi125489006) and *Capra hircus* (Goat, gi40950152, gi2575821). The illustration highlights a portion of alignment corresponding to probable transmembrane (TM) regions and the cytoplasmic loops. The residues in the predicted TM helices shown in the figure correspond to the mouse sequences. Conservation of preferred residues from the probable cholesterol binding sites resembling CRAC [VxxxxFxxxxxR] and CCM motif [FxxVxxFxxxR] are presented in red column. In MHC II, chain β, which has an overlapping CRAC/CCM motif has either Arg/Tyr (in yellow) occupying a position in between FxxxR and probably could also play an important role in cholesterol binding. Similarly, in chain α, conservation of a polar residue is highlighted in yellow (also see Table S1). The GxxxG motifs comprising of Gly residues are highly conserved (in green). At extreme right, helical wheels for the TM (chains α and β, top and bottom) clearly showed clustering of Gly residues (in gray). The residues at the termini of TM are shown with 'N' and 'C', respectively, for N- and C-terminus (in red).

Figure S2. Possible helix-helix packing of transmembrane helices of MHC II.

The TM helices of MHC II from chains, α (in red) and β (in blue) are shown in different possible helix-helix packing orientations i.e. right-handed, parallel and left-handed. The helices are represented as cylinders and the adjoin residues as coils. To ascertain the most probable helix-helix packing in TM, all the possible orientations (starting structures) are submitted for vacuum simulation for 10 ns. The final structures obtained at the end of the simulations converged to similar helix-helix packing.

Figure S3: Analysis of the specificity of NBD-cholesterol binding with TM-MHC-II. Exactly the same experiment was performed in the presence of increasing concentration of unlabelled cholesterol (▬) and unlabelled analogue (▬) (100 nM, 600 nM, 1.1 µM). The emission was measured at λ_{em} (520 nm). The data are plotted as F/F_0 vs cholesterol concentration. 'F_0' is the fluorescence intensity in absence of unlabeled cholesterol and 'F' is that in presence of it.

Supplementary Table

Table S1. Distances between residues at probable cholesterol binding sites and cholesterols.

Protein	Region of cholesterol[a]	Distance[b] (Å)	Residue conservation[c] (%)	Residue substitution[d]
V234:α	Tail	4.8Å	100	ns
F239:α	Rings	7.5Å	90	Phe→Leu
R245:α	Head	6.0Å	100	ns
I241:α	Rings	4.0Å	100	ns
Q242:α	Head	3.4Å	60	Gln→Arg/Lys
F240:β	Tail	6.3Å	100	ns
L243:β	Tail	3.2Å	30	Leu→Val
F246:β	Rings	4.3Å	90	Phe→Ile
R250:β	Head	4.6Å	80	Arg→Lys
I247:β	Ring	3.9Å	100	ns

[a] Based on the structure, the cholesterol molecule could be divided into three distinct regions: aliphatic tail, aromatic rings in the middle and the polar oxygen at the head.

[b] Minimum distance between heavy atoms of interacting residues (side chain) and cholesterol.

[c] Percentage conservation of residues from the alignment, see supplementary figure 1.

[d] Conservative substitution where the chemical properties are unaltered.

ns – No substitution as the residue is 100% conserved. Residues comprising the probable CRAC or CCM like motifs are in italics.

Table S2. Assessment of the assembled MHC II homology model by the checker programs.

Protein	Method	PDB ID/Model	Procheck[a]	WhatCheck[b]	Verify_3D[c]	Errat
MHC II	X-Ray	2IAD[d]	90.3	–3.915	96.64	81.84
ErbB2	NMR	2JWA[e]	77.0	–5.416	na	91.43
EphA2	NMR	2K9Y[e]	75.8	–5.638	na	96.30
MHC II	Homology	MHC II	80.0	–0.381	78.30	93.16

[a] φ/ψ percentage in core of Ramachandran plot.

[b] Structure Z-scores for chi-1/chi-2 rotamer normality. Positive values are better than average.

[c] Percentage of residues with average 3D-1D score > 0.2.

[d] 2IAD resolved at 2.40Å and has only extracellular-domain crystallized.

[e] TM helices from MHC II, chain α and β, was modeled based on template, 2K9Y and 2JWA, respectively. For NMR structures, only "MODEL 1" is considered.

na – Not available